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Unique isoform-specific properties of calsequestrin in the heart and skeletal muscle

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ABSTRACT

Calcium signaling in myocytes is dependent on the cardiac ryanodine receptor (RyR2) calcium release channel and the calcium buffering protein in the sarcoplasmic reticulum, cardiac calsequestrin (CSQ2). The overall properties of CSQ2 and its regulation of RyR2 have not been explored in detail or directly compared with skeletal CSQ1 and its regulation of the skeletal RyR1, with physiological ionic strength and Ca^{2+} concentrations. We find that there are major differences between the two isoforms under these physiological conditions. Ca^{2+} binding to CSQ2 is 50% lower than to CSQ1. Only ~30% of CSQ2 is bound to cardiac junctional face membrane (JFM), compared with ~70% of CSQ1 and the ratio of CSQ2 to RyR2 is only 50% of the CSQ1/RyR1 ratio. Chemical crosslinking shows that CSQ2 is mostly monomer/dimer, while CSQ1 is mostly polymerized. In single channel lipid bilayer experiments, CSQ2 monomers and/or dimers increase the open probability of both RyR1 and RyR2 channels, while CSQ1 polymers decrease the activity of RyR1. We speculate that CSQ2 facilitates high rates of Ca^{2+} release through RyR2 during systole, while CSQ1 curtails RyR1 opening in response to a single action potential to maintain Ca^{2+} and allow repeated Ca^{2+} release and graded activation with increased stimulation frequency.

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1. Introduction

Excitation contraction (EC) coupling is the mechanism linking depolarization of the surface membrane to Ca^{2+} release from the sarcoplasmic reticulum (SR) and the process which initiates cardiac and skeletal muscle contraction. Ca^{2+} release during EC coupling is triggered by a surface/transverse-tubule action potential. The SR Ca^{2+} release channel – the ryanodine receptor (RyR) – has recruited a surface membrane Ca^{2+} channel (the dihydropyridine receptor) as a voltage sensor to detect the action potential, and recruited the Ca^{2+} binding protein calsequestrin (CSQ) to sense the environment inside the SR. Thus the RyR forms the hub of a giant macromolecular complex with transmembrane, luminal and cytoplasmic components. Luminal interactions with CSQ communicate the Ca^{2+} load in the store to the RyR [1–4], but the molecular interactions underlying this communication are not well understood in either skeletal muscle or cardiac myocytes. There is an emerging interest in the role of cardiac CSQ since mutations in the protein, or indeed its absence, can lead to catecholaminergic polymorphic ventricular tachycardia [5,6].

Two isoforms of CSQ expressed in muscle are encoded by different genes [7,8]. CSQ1 or "skeletal" CSQ is the only isoform expressed in fast-twitch skeletal muscle and is the major isoform in slow-twitch skeletal muscle [9,10]. CSQ2, "cardiac" CSQ, is the sole CSQ isoform expressed in cardiac muscle and is a minor transcript in slow-twitch skeletal muscle. The two isoforms of CSQ differ mainly in their C-terminal region with CSQ2 having an extended C-terminus (residues 367–391, of which 71% are acidic). Both CSQ isoforms can act as a luminal Ca^{2+} sensor for the RyR [1–4]. However CSQ1 and CSQ2 have dissimilar Ca^{2+} binding capacities [11] and may undergo different structural changes over a $[Ca^{2+}]$ range of 0–1 mM [12]. Thus it is possible that CSQ regulates the RyR in different ways in the two muscle types. CSQ1 inhibits RyR1 at a physiological luminal $[Ca^{2+}]$ of 1 mM [13] or activates RyR1 when added at lower luminal Ca^{2+} [14]. Native cardiac RyR2 channels are more active in the presence of CSQ2 at low luminal Ca^{2+} [14] and at luminal Ca^{2+} concentrations between 250 μM and 1 mM [4]. In contrast CSQ2 has been reported to also inhibit RyR2 when luminal Ca^{2+} concentration is less than 1 mM (20 μM) [2,15]. The direct effect of dissociating and reassociating CSQ2 with RyR2 at a $[Ca^{2+}]$ within the physiological range (250 μM–1 mM) has not been evaluated. Nor have the molecular interactions between the two proteins been directly compared, although the data summarised above suggest that there may be tissue-specific differences between the effects of CSQ1 and CSQ2 on RyR1 or RyR2 respectively.

In this manuscript we show that the Ca^{2+}-dependence of CSQ polymerization and CSQ's ability to regulate the RyR vary sub-

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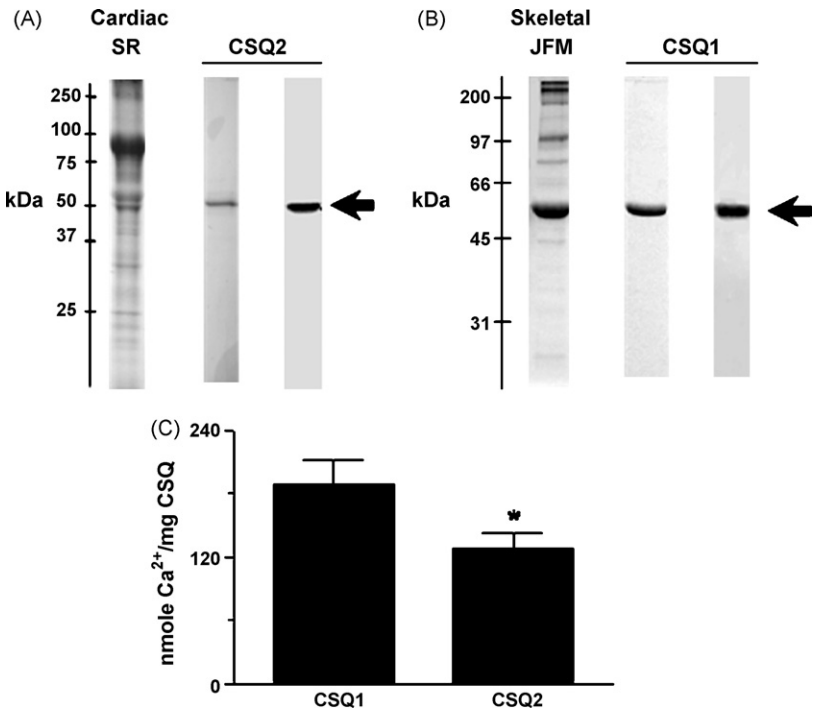

Fig. 1. Purification of CSQ and Ca^{2+} binding of sheep cardiac and rabbit skeletal CSQ. (A and B) Sheep cardiac SR (A; lane 1) and rabbit skeletal JFM (B; lane 4) used to purify CSQ2 (A; lane 2, arrow) and CSQ1 (B; lane 5, arrow) at ~50 kDa and ~55 kDa, respectively, on 10% SDS-PAGE. The identity of CSQ1 and CSQ2 was confirmed by immuno-blot using anti-CSQ2 (A; lane 3) and anti-CSQ1 (B; lane 6) antibodies. (C) $^{45}Ca^{2+}$ binding to isolated and purified rabbit skeletal CSQ1 and sheep cardiac CSQ2 in the presence of 1 mM Ca^{2+}.

stantially between the CSQ and RyR isoforms. We suggest that the different properties of the two CSQ isoforms are consistent with the different properties of the Ca^{2+} stores in skeletal and cardiac muscle and present a model describing in part, CSQ regulation of RyR1 and RyR2.

2. Materials and methods

2.1. SR vesicle isolation

Skeletal SR vesicles were prepared from New Zealand white rabbits [16] and cardiac SR vesicles from sheep heart [17]. The two different preparations were used for convenience and because we have an extensive data base on RyR1 from rabbit and RyR2 from sheep. There are no major differences between the responses of RyR1 or RyR2 channels from different species to most major ligands such as cytoplasmic Ca^{2+}, Mg^{2+} or ATP. Furthermore, protein sequence identity between CSQ from different mammalian species is ≥91% for CSQ1 and for CSQ2, while sequence similarity is ≥96% for CSQ1 or ≥95% for CSQ2. Early studies by several groups found that CSQ is the only Ca^{2+} binding protein in the SR of skeletal and cardiac muscle that is present in significant quantities (e.g. [18]). Similarly, $^{45}Ca^{2+}$ overlay and stains-all staining (both used to indicate Ca^{2+} binding proteins) show that CSQ1 or CSQ2 are the only significant SR Ca^{2+} binding proteins in our skeletal muscle and heart preparations (data not shown). Additional Ca^{2+} binding proteins were not detectable by these means.

2.2. Purification of CSQ from skeletal and cardiac muscle

Rabbit skeletal CSQ1 was purified from junctional face membrane (JFM) as described in [19]. Sheep cardiac CSQ2 was purified by gel elution [20] from large vertical slab SDS polyacrylamide gels using a denaturing buffer [21] using an 8.5% resolving gel and a 4% stacking gel. SDS-PAGE and Western blots of purified CSQ1 and CSQ2 are shown in Fig. 1A and B. Sheep cardiac CSQ2 was used in all experiments except for some of those in Fig. 6B.

2.3. Human recombinant CSQ2 expression

Human recombinant CSQ2 was expressed as His fusion proteins and expressed as our standard expression protocol (see [19]). Following sedimentation, cells were resuspended in a solution containing 50 mM NaH_2PO_4, 10 mM imidazol, 10 mM β-mercaptothenaol (pH 8.0) and disrupted using a French press. Following centrifugation at ~5000 × *g*, disrupted cells were incubated with a Ni-NTA Agarose matrix and washed with 4 × 15 volumes of 50 mM NaH_2PO_4, 20 mM imidazol, 10 mM β-mercaptothenaol (pH 8.0). CSQ2 was eluted in a buffer containing 50 mM NaH_2PO_4, 500 mM imidazol, 10 mM β-mercaptothenaol (pH 8.0). CSQ2 was dialyzed against a buffer containing 20 mM Mops, 150 mM NaCl and 1 mM $CaCl_2$ (pH 7.4) and stored in small aliquots at −70 °C.

2.4. SDS-PAGE and Western blot

SDS-PAGE and Western blot are described in [21,22].

2.5. Chemical crosslinking

To visualize the degree of association of CSQ (i.e. dimerization or polymerization) at various [Ca^{2+}]s, 100 μM purified skeletal or cardiac muscle CSQ was incubated at room temperature for 4–18 min at a [Ca^{2+}] of 1 mM in the presence of 150 mM NaCl and 20 mM MOPS. The samples were further incubated with the crosslinker 20 mM *1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Hydrochloride* (EDC) and 50 mM *N-hydroxysuccinimide* (NHS) for 15 min at room temperature. EDC is a zero-arm length crosslinker, which directly couples carboxyls to primary amines. There is no spacer between coupled molecules. Thus, EDC only allows crosslinking

of molecules which are physically interacting (such as dimers and polymers). The reaction was stopped by the addition of 1:1 SDS-PAGE sample buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 10% sucrose, 2.5% SDS, 5 mM DTT, and 0.02% bromophenol blue) and loaded on 7.5% or 4–15% gradient gel immediately for electrophoresis and subsequent immunoblotting. Images of SDS gel electrophoresis were taken by Genesnap 6.0 and band density was quantified by Genetools Analysis Software 3.0 (Synoptics, Cambridge, UK).

2.6. ^{45}Ca binding assay

The Ca^{2+} binding capacity of CSQ was determined using a $^{45}Ca^{2+}$ spin dialysis binding assay [23].

2.7. Single channel recording and analysis

Artificial planar bilayers separating two baths (*cis* and *trans*) were formed as previously described [13,19]. SR vesicles (50 μg) were added to the *cis* solution so that the cytoplasmic surface of the SR and RyRs faced the *cis* solution after incorporation into the lipid bilayer. For SR vesicle incorporation, the solution compositions were as follows: *cis*: 230 mM CsMS, 20 mM CsCl, 1 mM $CaCl_2$, and 10 mM TES (pH 7.4); and *trans*: 30/230 mM CsMS, 20 mM CsCl, 1 mM $CaCl_2$, and 10 mM TES (pH 7.4). After incorporation of a channel, *trans* [Cs^+] was raised from 50 mM to 250 mM with the addition of 200 mM CsMS and the *cis* solution was altered by the addition of 4.5 mM BAPTA (free [Ca^{2+}] = 100 nM) and 2 mM ATP. Endogenous CSQ was dissociated from the RyR/Triadin/Junctin complex by high Ca^{2+} or low Ca^{2+} or high ionic strength [13]. Perfusion of the *trans* chamber to restore control conditions, or adjustment of Ca^{2+} to desired concentrations (1 mM) was carried out prior to the addition of 16 μg/ml CSQ. Single channel parameters were obtained using the Channel 2 program (developed by P.W. Gage and M. Smith, John Curtin School of Medical Research, Canberra, Australia). Channel activity was assessed from 30 s to 180 s records, by measuring open probability (P_o) directly using threshold discrimination or indirectly from the fractional mean current (I'_F). The fractional mean current (I'_F), is the average of all data points obtained during a recording period, divided by the maximum single channel current. I'_F is approximately equal to the P_o and relative P_o = relative I'_F. For simplicity, data obtained with both measurements are presented as either the P_o or relative P_o. All electrical potentials are expressed here using standard physiological convention (i.e. cytoplasmic side relative to the luminal side at virtual ground). Single channel recordings were obtained at +40 mV and −40 mV, at 23 ± 2 °C.

2.8. Statistics

Average data are presented as mean ±SEM. The significance of differences between values was tested using a Student's *t*-test for paired data or by the non-parametric sign test [24]. A *P* value of <0.05 was considered to be significant.

3. Results and discussion

3.1. Ca^{2+} binding capacity of CSQ

$^{45}Ca^{2+}$ binding to purified CSQ1 and CSQ2 (Fig. 1A and B) was compared at the resting luminal [Ca^{2+}] of 1 mM. Skeletal CSQ1 bound on average 1.5-fold more Ca^{2+} than cardiac CSQ2 (Fig. 1C). This is consistent with atomic absorption data showing rabbit CSQ1 binding 50% more Ca^{2+} than canine CSQ2 with 1 mM Ca^{2+} [11]. That less Ca^{2+} binds to CSQ2 is somewhat surprising, considering CSQ2 contains an extended C-terminal tail when compared with CSQ1,

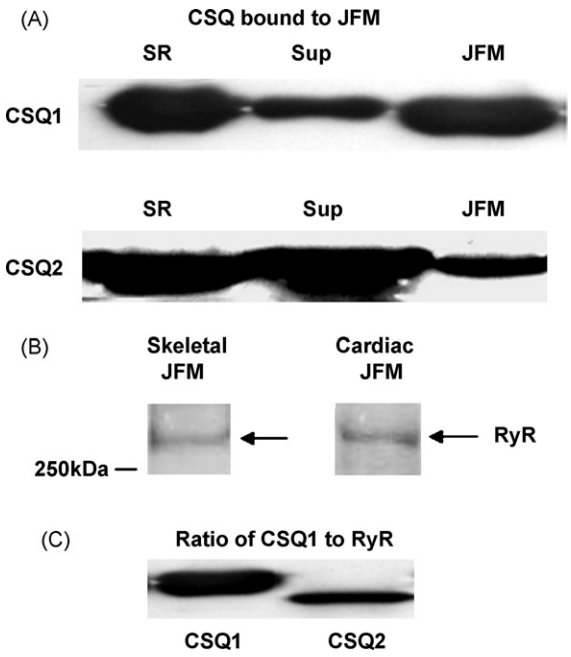

Fig. 2. Less CSQ2 is associated with cardiac JFM than CSQ1 associated with skeletal JFM. (A) Western blots of a skeletal (upper) and cardiac (lower) preparations. CSQ in SR before solubilization (lane 1) and in the supernatant (lane 2) or membrane pellet following solubilization and centrifugation (lane 3). (B) SDS-PAGE of 3 μg of skeletal JFM and 7 μg of cardiac JFM, which yielded equivalent amounts of RyR. (C) Western blots of the amounts of CSQ1 (lane 1) and CSQ2 (lane 2) associated with equal amounts of RyR1 and RyR2, respectively. The anti-CSQ1 and anti-CSQ2 antibodies were titrated to produce equivalent immunostaining of equal amounts of CSQ1 and CSQ2.

and the extended tail is composed of >70% acidic residues. Since the C-terminal tail is thought to form a Ca^{2+} binding pocket in the CSQ polymer [25] the larger number of acidic residues might be expected to confer greater Ca^{2+} binding. This is clearly not the case.

3.2. CSQ association with the RyR complex

In rabbit skeletal SR, the majority of CSQ1 (~70%) is in a polymer form and is associated with the JFM [3,19,25]. The association between CSQ2 and cardiac JFM has not previously been determined and the data in Fig. 2A indicate that it is substantially less than in skeletal muscle. In this experiment, SR vesicles were solubilized with 0.5% Triton X-100 and 1 mM Ca^{2+} and then centrifuged to separate the membrane-bound proteins in the pellet from soluble proteins in the supernatant. SDS-PAGE and immuno-blot revealed that on average only 29.9 ± 0.24% (n = 5) of CSQ2 was in the cardiac JFM pellet, with the remaining 70.1 ± 2.4% contained in the supernatant. Skeletal SR was examined at the same time and 69.9 ± 1.9% of CSQ1 (n = 5) was membrane bound and 30.1 ± 1.9% was found in the supernatant.

The smaller fraction of CSQ2 associating with cardiac JFM (and hence the RyR2) could be explained by either a similar amount of CSQ2 bound to RyR2 (but fewer RyR2 channels) or by less CSQ2 associated with each RyR2. Thus the ratio of CSQ to RyR was examined by loading 3 μg of skeletal JFM and 7 μg of cardiac JFM onto gels (amounts which yielded similar densities of RyR1 and RyR2 on SDS-PAGE, Fig. 2B). For immunoblot, anti-CSQ1 and anti-CSQ2 antibodies were also titrated to establish concentrations which produced the same staining density for the equal amounts of CSQ1 and CSQ2 indicated by quantification of band density. Scanning the immunoblots (Fig. 2C) revealed that the CSQ2/RyR2 ratio was only half the CSQ1/RyR1 ratio. Therefore the smaller amount of CSQ2

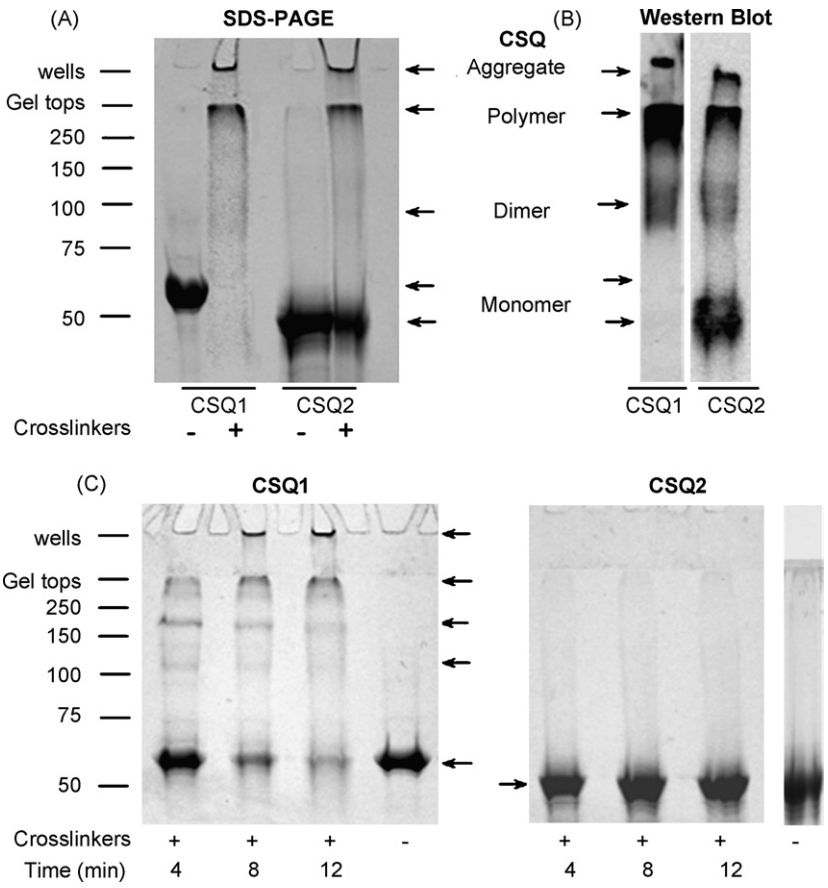

Fig. 3. CSQ2 is mostly in a monomer or dimer form with 1 mM Ca^{2+}, while CSQ1 is polymerized. (A) 7% SDS-PAGE of purified CSQ1 (lanes 1 and 2) and CSQ2 (lanes 3 and 4), before (lanes 1 and 3) and after (lanes 2 and 4) crosslinking at physiological ionic strength (150 mM NaCl) and luminal [Ca^{2+}] (1 mM). CSQ is monomeric before exposure to crosslinker. After crosslinking for 18 min, all CSQ1 appeared to be polymerized, while much CSQ2 was not polymerized. (B) Western blot of crosslinked CSQ from lanes 2 and 4 in (A) revealed small amounts of dimer and tetramer in the CSQ1 preparations as well as the higher molecular weight polymers (lane 1) and small amounts dimer and tetramer in CSQ2 (lane 2). (C and D) CSQ exposed to crosslinker for 4, 8 and 12 min and run on 7% SDS-PAGE. CSQ2 (right) was less polymerized than CSQ1 (left) after 4 (lane 1), 8 (lane 2) and 12 min (lane 3) and without crosslinker (lane 4). Molecular weight markers are shown to the left. The concentration of CSQ1 and CSQ2 used for crosslinking was 100 μM as described in the text.

associated with cardiac JFM (Fig. 2A) is due to less CSQ2 associated with the RyR2.

The number of CSQ molecules that can associate with the RyR is determined by two factors. (1) The numbers of triadin and junctin binding sites for CSQ and (2) by the degree of polymerization of CSQ, since the number of molecules associating with the RyR (via triadin and junctin) must increase with the length of the polymer. Previous crosslinking data showed that, with 2 mM free Ca^{2+}, CSQ2 is mostly monomer with some dimer [26], in contrast to CSQ1 which is mostly polymerized [3]. Since the degree of polymerization may be CSQ concentration-dependent and the CSQ concentrations of 10–20 μM in data reported to date [3,26] are considerably lower than concentrations of ~100 μM found in the SR,[2] we compared crosslinking of CSQ1 and CSQ2 using higher CSQ concentrations of ~100 μM.

3.3. Polymerization of CSQ1 and CSQ2

CSQ1 and CSQ2 were crosslinked with zero-arm carboxyl-amine crosslinkers *1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Hydrochloride* (EDC) and *N-hydroxysuccinimide* (NHS) using 100 μM CSQ with 1 mM Ca^{2+} and 150 mM NaCl. There was a remarkable difference between the extent of crosslinking of CSQ1 and CSQ2 after 18 min exposure to the crosslinkers, with all CSQ1 present as high molecular weight polymers or as larger aggregates retained in the gel wells (Fig. 3A). Some dimer and tetramer forms of CSQ1 can be seen in the Western blots, but there is no evidence of monomers (Fig. 3B), i.e. 100% CSQ1 was in present as dimer or higher molecular weight form, with most of the protein forming polymers. In marked contrast, the majority of CSQ2 was monomeric with only a small amount present as a dimer or higher molecular weight polymer after exposure to crosslinker for 18 min. Since non-specific crosslinking may have occurred during the 18 min incubation with this high concentration of CSQ, briefer exposure times of 4, 8 and 12 min were used (Fig. 3C). Cardiac CSQ2 was not significantly crosslinked after these briefer exposures. Again in marked contrast to CSQ2, most CSQ1 was crosslinked with dimers, tetramers and higher molecular weight forms apparent after only 4 min exposure and ~90% of the protein crosslinked after 12 min. With incubation times >4 min, a significant amount of CSQ1 was retained in the wells at the top of the gel and was not separated by the resolving gel. We assume that this constitutes high molecular weight CSQ1 polymers which could not run into the gel. Indeed, CSQ1 (but not CSQ2) precipitation was visualized in the test tube with crosslinking times >10 min. These results indicate that CSQ2 is not significantly polymerized when physiological concentrations of the monomer (100 μM) were exposed to 1 mM Ca^{2+} in a solution of physiological ionic strength. The lack of significant polymerization would

[2] Calculated from the fact that ~8 mg of CSQ1 is isolated from 100 g of muscle and that 4% of the fiber volume is occupied by terminal cisternae [27,28], the specific gravity of muscle is 1.06 [29] and the MW of CSQ is 40 kDa [30]. We assume ~50% loss of protein during the multi-step purification.

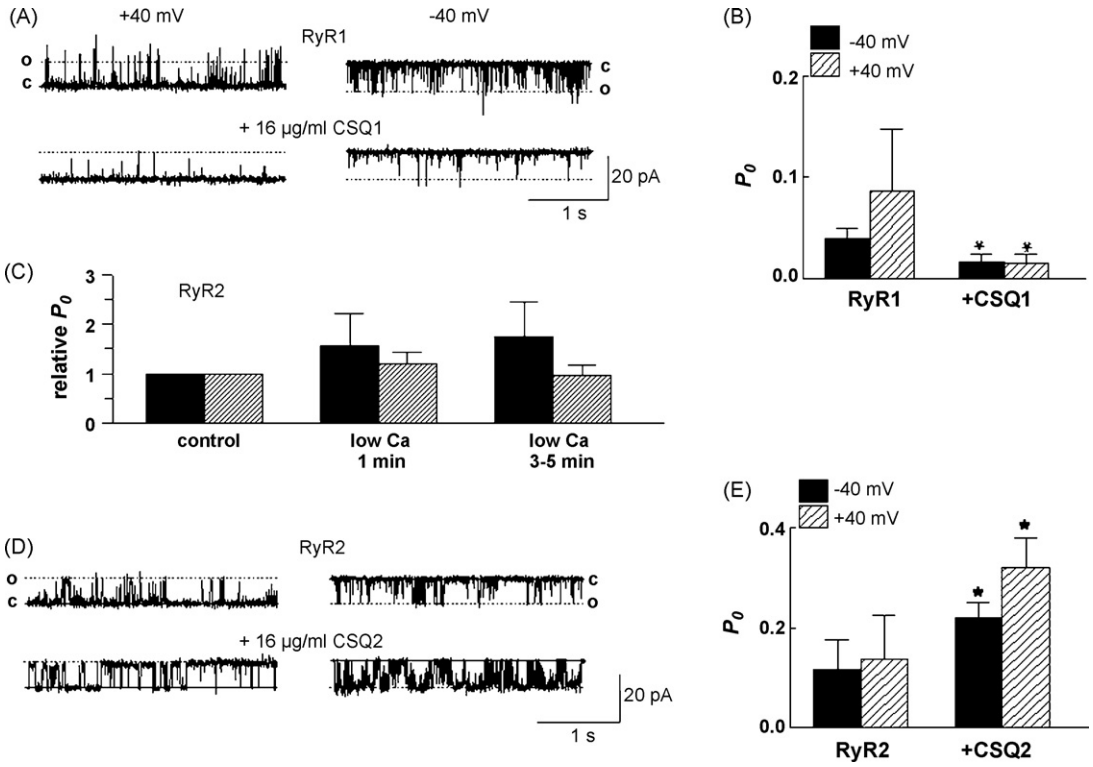

Fig. 4. CSQ1 inhibits RyR1, while CSQ2 activates RyR2 in the presence of 1 mM luminal Ca^{2+} after exposure to low Ca^{2+}. (A) Record of 3 s of single skeletal RyR1 channel activity before (control) and after addition of CSQ1. Channel opening is upward from zero current (c) to maximum open conductance (o) at +40 mV (left panel) and downward at −40 mV (right panel) with 500 nM *cis* Ca^{2+}. (B) Average P_o for 30 s records from RyR1 at +40 mV and −40 mV under control conditions and after addition of CSQ1 ($n = 6$). (C) Relative open probability (P_o) of cardiac RyR2 channels after exposure to low luminal Ca^{2+} (10 μM) for 1 min or for 3–5 min, with 500 nM *cis* Ca^{2+} (filled bins $n = 7$) or 100 μM *cis* Ca^{2+} (hatched bins $n = 4$). (D and E) Identical to (A and B) except that data are recorded from RyR2 before and after addition of CSQ2 ($n = 8$). In (B and E), asterisks (*) indicate significant differences from channels before CSQ addition.

explain the small amounts of CSQ2 associated with RyR2 in Fig. 2 above.

It could be argued that the differences between CSQ1 and CSQ2 reflect differences in the ways that the two proteins behave with the chemical crosslinkers. However these results are remarkably similar to results showing a failure of CSQ2 to form polymers with physiological Ca^{2+} and ionic strength obtained with the very different light scattering/turbidity techniques [4,26]. Results from these studies support our observation that CSQ2 does not form a polymer in the presence of 150 mM Na^+ and 1 mM Ca^{2+}. Visual inspection (our unpublished observations) and light scattering [4,26] suggests that CSQ2 can either aggregate or form polymers if Ca^{2+} is increased to >5–10 mM, however this is significantly higher than the 0.5–1.0 mM in the lumen of the cardiac SR [31,32]. CSQ2 can also form a polymer at 1 mM Ca^{2+} if ionic strength is reduced below physiological levels [5,26]. FRET studies indicating interactions between CSQ2 molecules within living fibroblast are explained by polymerization [31] do not necessarily contradict our conclusions, since the results could equally well reflect dimer formation. The light scattering observations [4,26], together with the data presented in Figs. 2 and 3, lead to the conclusion that CSQ2 does not form a polymer in the presence of a physiological Ca^{2+} concentration of 1 mM and ionic strength of 150 mM.

It is also interesting to note that most electron micrographs revealing clear examples of CSQ forming a linear polymer are of skeletal muscle (e.g. [33–35]. The structure of CSQ in cardiac muscle is less clear (e.g. [36]) and this could be due either to a lack of polymerization or to the more crowded architecture of the terminal cisternae in myocytes. There is material condensed near the junctional face membrane, however the micrographs do not reveal whether the condensations are linear CSQ polymers, CSQ dimers or CSQ monomers/dimers which have accumulated near the membrane.

The failure of CSQ2 to polymerize with 1 mM Ca^{2+} may be a function of, or alternatively be a cause of, its lower Ca^{2+} binding capacity. It is possible that the longer and more highly charged C-terminal tail of CSQ2 sterically hinders polymerization. Since polymerization involves the formation of the C-terminal tail into a Ca^{2+} binding pocket [25], interference with polymerization would also reduce Ca^{2+} binding.

3.4. Regulation of the RyR1 by CSQ1 and RyR2 by CSQ2 with 1 mM luminal (trans) Ca^{2+}

RyR1 regulation by CSQ1 depends on its association with CSQ1 polymers (not monomers) ([3] and Fig. 5D and E). Since CSQ2 does not polymerize under resting conditions, regulation of RyR2 by CSQ2 may differ from the regulation of RyR1 by CSQ1. This possibility was examined by dissociating endogenous CSQ from RyR channels and then examining the effects of adding exogenous CSQ back to the channels. CSQ was dissociated in two ways, either by exposure to low luminal Ca^{2+} or to high luminal ionic strength [3,13]. In the first case native RyR channels were exposed to a solution with no added Ca^{2+}, containing 1 mM BAPTA ($Ca^{2+} < 1$ nM) for >5 min, then the luminal Ca^{2+} was restored to 1 mM and exogenous CSQ added. Exposure to low luminal [Ca^{2+}] depolymerizes CSQ1 and dissociates all but a residual monomer from the RyR1 complex [3], seen as an increase in RyR activity due to the removal of CSQ1 inhibition. A monomer is thought to remain because CSQ binding to triadin is retained with low Ca^{2+} [20,37]. The residual CSQ1 monomer does not regulate RyR1 or its sensitivity to luminal

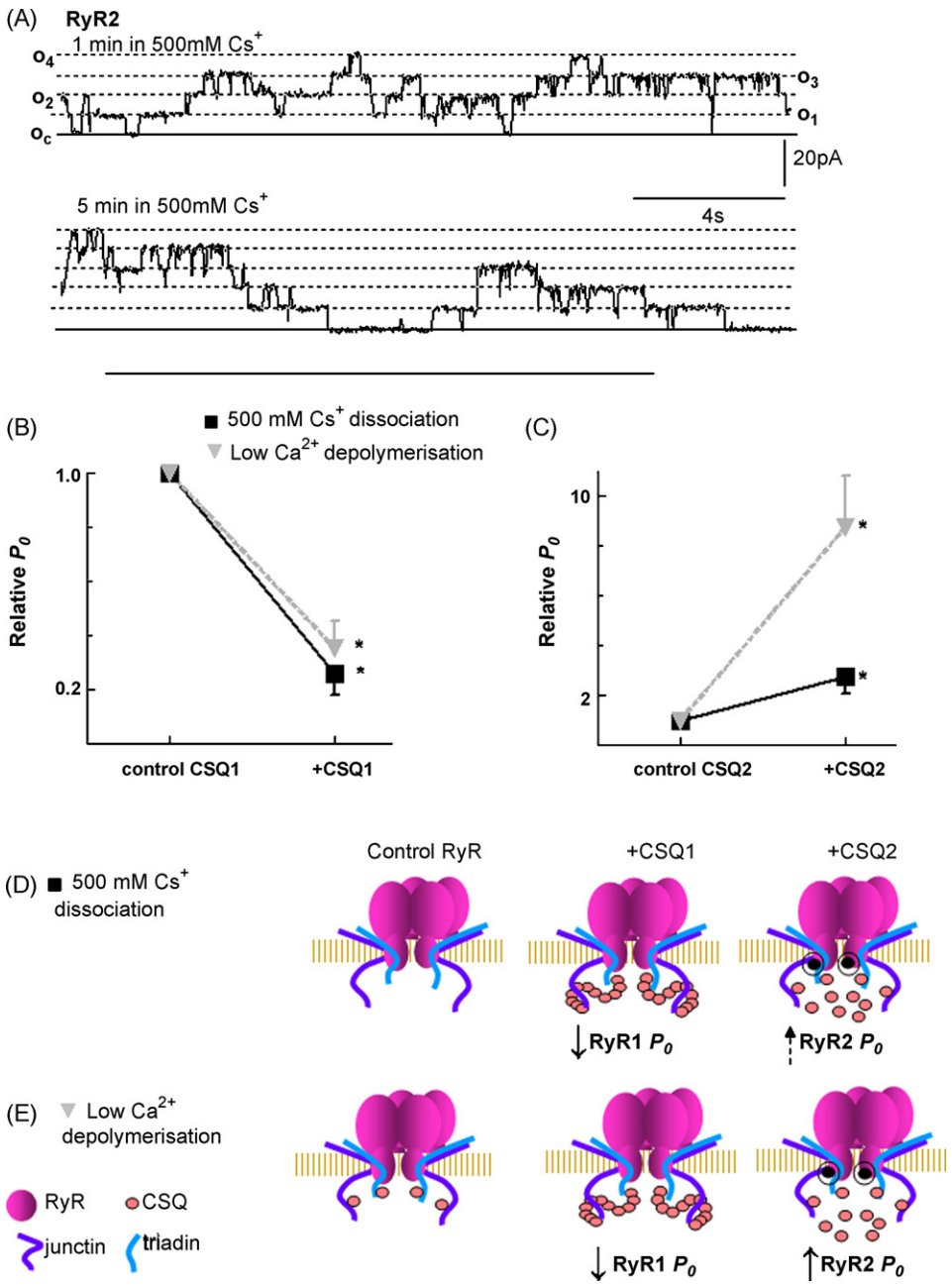

Fig. 5. CSQ1 inhibits RyR1, while CSQ2 activates RyR2 in the presence of 1 mM luminal Ca^{2+} after exposure to high ionic strength. (A) Recording from a bilayer containing 5 RyR2 channels at +40 mV, 1 min after exposure to 500 mM Cs^+ (upper) and the subsequent increase in channel closures (which we speculate is CSQ2 monomer dissociation) 5 min after exposure to 500 mM Cs^+ (lower record) with 1 mM *trans* Ca^{2+} and 500 nM *cis* Ca^{2+}. (B and C) Average relative P_o for combined data obtained at +40 mV and −40 mV (n = 8–9) with 1 mM *trans* Ca^{2+} from RyR1 (B) and RyR2 (C). Effect of adding exogenous CSQ (after restoration of Cs^+ to 250 mM) to RyRs stripped of CSQ with 500 mM Cs^+ (■) or RyRs after CSQ depolymerization during exposure to low Ca^{2+} (▼). Control P_o values for channels are 0.18 ± 0.11 for (■) and 0.09 ± 0.05 for (▼). Data for CSQ1/RyR1 for 500 mM Cs^+ dissociation (B) are replotted in part from [13] and included for ease of comparison. With RyR2 (C), control P_o for channels is 0.13 ± 0.04 for (■) and 0.14 ± 0.06 for (▼). Asterisks (*) indicate significant differences from channels before CSQ addition. (D–E) A model showing the different effects of exposing RyRs (RyR1 or RyR2) to high ionic strength (D, 500 mM Cs^+, which disrupts CSQ ionic interactions with triadin and junctin and leaves almost no CSQ attached, left upper panel) or to low Ca^{2+} (E, ≤100 μM Ca^{2+}, which causes CSQ depolymerization and leaves a CSQ monomer attached to triadin and junctin) and the effect of then adding CSQ1 to RyR1 (middle panel) or CSQ2 to RyR2 (right panel) once the usual [Ca^{2+}] of 1 mM and ionic strength of 250 mM are restored. CSQ1 polymers (middle panel) and CSQ2 monomer (right panel) are shown associating with the RyR via triadin and junctin) and changes in RyR1 open probability due to CSQ association is depicted under each panel. A possible direct binding site on the RyRs for CSQ2 (whose binding may be reversible at 1 mM Ca^{2+}) is indicated by the open circles on the RyRs in the right panel.

[Ca^{2+}] [3]. However, since it is possible that the remaining monomer may influence the response of RyR2 to exogenous CSQ2, the effect of exogenous CSQ was also examined in the absence of residual monomers. This was achieved using high ionic strength to dissociate >95% of CSQ (including residual monomers [3,13]), while maintaining luminal Ca^{2+} at 1 mM. Parallel experiments were performed with CSQ1 and CSQ2 to compare the isoforms under the same conditions.

(a) *CSQ addition to RyRs in the presence of 1 mM luminal Ca^{2+} after exposure to low Ca^{2+}.*

It was of interest to examine the effects of CSQ on the RyR when luminal Ca^{2+} was lowered and then restored to 1 mM since exposure to low Ca^{2+} and then restoration of 1 mM Ca^{2+} partly mimics the fall and rise of luminal Ca^{2+} that might occur during a skeletal contraction cycle or during muscle fatigue and subsequent recovery. CSQ1 may depolymerize during activity

and repolymerize during rest [3,38]. The concentrations of Ca^{2+} that we used were lower than those achieved *in vivo* and were used in order to obtain maximal CSQ dissociation. CSQ1 was added at 16 μg/ml to maximally inhibit CSQ-deficient RyR1 [13 and Fig. 6 below].

Addition of 16 μg/ml CSQ1 to CSQ1-deficient RyR1s inhibited the channels by ~50% (Fig. 4A and B). This fall in activity was similar to that seen with CSQ1 addition after exposure to 100 nM or 100 μM Ca^{2+} [3] or after using high Cs^+ to dissociate all CSQ [13]. Control experiments have shown that exogenous CSQ1 does not alter RyR1 activity unless endogenous CSQ1 has been removed [13]. Since CSQ1 is a polymer with 1 mM Ca^{2+} (Fig. 2 above and [3]), our model (Fig. 5D and E) is that exogenous CSQ1 forms a polymer by binding to the residual endogenous CSQ monomer which remains attached to the RyR1 complex after depolymerization in low Ca^{2+}. The inhibition is consistent with the concept that the CSQ1 polymer reduces the activity of RyR1.

The decrease in activity when CSQ1 was added to CSQ1-deficient RyR1 was a mirror image of that seen when CSQ1 was dissociated from native RyR1 (ie an increase in activity when the CSQ1 polymer depolymerizes after >3 min exposure to CSQ1 dissociating solutions [3,13]). Given that most of CSQ2 would not be in a polymer form (Fig. 3), this delayed change in RyR2 channel activity due to CSQ depolymerization was not expected to occur with a native RyR2. Indeed, there was no significant change in RyR2 activity within 1 min of the drop in luminal Ca^{2+} from 1 mM to 10 μM, or after 3–5 min (Fig. 4C), when depolymerization would have been complete if it had occurred. The result was consistent with concept that most CSQ2 was monomeric in the presence of 1 mM Ca^{2+} and remained so during exposure to lower Ca^{2+} concentrations. An extension of this argument was after luminal Ca^{2+} was restored to 1 mM, addition of exogenous CSQ2 to CSQ-deficient RyR2 would not alter channel activity, since it would not polymerize. Contrary to expectations, and in marked contrast to the fall in activity in the skeletal system, the average P_o rose ~2-fold with a delay of ~3 min after adding 16 μg/ml CSQ2 to the *trans* chamber (Fig. 4C–E). It is notable that the changes in activity were not voltage-dependent in either skeletal or cardiac preparations since similar average changes are apparent at +40 mV and −40 mV.

How does the exogenous CSQ2 activate RyR2, if the triadin/junctin binding sites are occupied by a CSQ2 monomer and CSQ2 does not polymerize? Among several possibilities are that exogenous CSQ2 binds reversibly to RyR2 and activates the channel, in the same way as CSQ1 activates purified RyR1 [13,39] at the same time as the endogenous CSQ2 remained irreversibly bound to triadin and junctin, with luminal Ca^{2+} ≤1 mM. In a bilayer experiment, the loosly (reversibly) bound CSQ2 would dissociate when the cardiac SR vesicles fused with the lipid bilayer and luminal portion of the SR was exposed to the 1 ml *trans* chamber with a very low concentration of endogenous CSQ2. Thus these binding sites would be available when the exogenous CSQ was added. If this was the case, activation may also be seen if CSQ2 was added to RyR2 that had not been treated to dissociate endogenous CSQ2. In one control experiment, CSQ2 was added to the luminal side of RyR2 channels that had not been treated to dissociate endogenous CSQ2 and an increase in activity was seen (data not shown). Whatever the mechanism underlying the increase in activity, it appears that the soluble CSQ2 in the cardiac SR may increase activity in RyR2 channels. This role of CSQ2 in increasing the open probability of RyR2 in the heart is very different from the decrease in RyR1 activity induced by CSQ1 in fast twitch skeletal muscle.

(b) *CSQ addition to RyRs after endogenous CSQ dissociation with high ionic strength.*

Exposure to 500 mM Cs^+ in the *trans* (luminal) solution leads to an increase in RyR1 channel activity when CSQ1 dissociates after ~5 min [3,13], due to the removal of CSQ1 regulation. In contrast to RyR1, RyR2 channel activity fell after 5 min in the high ionic strength solution (Fig. 5A). We speculate that this delayed decrease in RyR2 activity occurred upon CSQ2 monomer dissociation, since reassociation of CSQ2 with RyR2 channels (after restoring ionic strength to 250 mM) led to an increase in channel activity (Fig. 5C). This was again in contrast to the fall in RyR1 activity in a parallel experiment (Fig. 5B).

The opposing effects on RyR1 and RyR2 are presumably both caused by the interaction of CSQ with the channel complexes, which is illustrated in our model in Fig. 5D and E. The results obtained with exogenous CSQ2 addition after endogenous CSQ2 dissociation with high ionic strength, were the same as the results obtained after CSQ2 exposure to low Ca^{2+} (Fig. 5B and C), indicating that the CSQ2 monomer retained after low Ca^{2+} treatment [3] did not influence the response of the RyR to exogenously added CSQ2. Thus, unlike CSQ1 (see section a above) CSQ2 is unable to form polymers and therefore cannot associate with the residual CSQ2 monomer. This again points to part of the activation by exogenously added CSQ2 occurring through a separate, perhaps direct, interaction with the RyR2 protein. An increase in activity with CSQ2 addition after high ionic strength dissociation may also be due to a direct interaction with RyR2 and to re-association with junctin and triadin. Some RyR regulatory effect of re-association of CSQ2 with triadin and junctin is postulated because there was a decline in activity during exposure to the high ionic strength solution causing CSQ2 dissociation from these proteins.

The changes in RyR1 and RyR2 activity are time-locked to the addition of CSQ1 or CSQ2. There are no equivalent changes in RyR1 or RyR2 activity if exogenous CSQ is not added to the *trans* solution. Channel activity remains constant until CSQ is added and then changes after 2–3 min–indicating that the change in activity is not an intrinsic time-dependent property of the RyR channels. Further evidence that the changes are not an intrinsic property of the RyR channel is the opposing changes in RyR1 activity produced by addition of CSQ1 or CSQ2 described below.

The illustration in Fig. 5D summarizes our model of the difference between stripping CSQ from RyRs with high ionic strength (almost no CSQ remaining attached to triadin and junctin) and of dissociating CSQ multimers with low Ca^{2+} treatment (whereby monomers remain attached to triadin and junctin) and the effect of subsequent addition of CSQ. Addition of CSQ1 to RyR1 with 1 mM luminal Ca^{2+} results in CSQ1 polymer reassociation with the channel complex and subsequent channel inhibition. Addition of CSQ2 to RyR2 with 1 mM luminal Ca^{2+} results in CSQ2 monomer association with the channel complex, and subsequent channel activation, by mechanisms not yet defined.

3.5. The concentration-dependence of CSQ1 and CSQ2 action

The concentration of CSQ1 that we routinely use is 16 μg/ml [3,13,20] as opposed to previous reports of CSQ2 which has been used at 0.5–5 μg/ml [2,4]. The difference between the activation of RyR2 by 16 μg/ml CSQ2 reported here and a previous inhibition reported by [2] may have been due to the different concentrations used. The concentration-dependence of CSQ effects on RyR channels is summarized in Fig. 6. CSQ1 inhibits RyR1 over a wide range of 0.8 μg/ml to 80 μg/ml (Fig. 6A), while CSQ2 activates RyR2 over a range of 5 μg/ml to 30 μg/ml, with activation maximal at 5 μg/ml (Fig. 6B). The data for the experiments with 5 μg/ml to 30 μg/ml

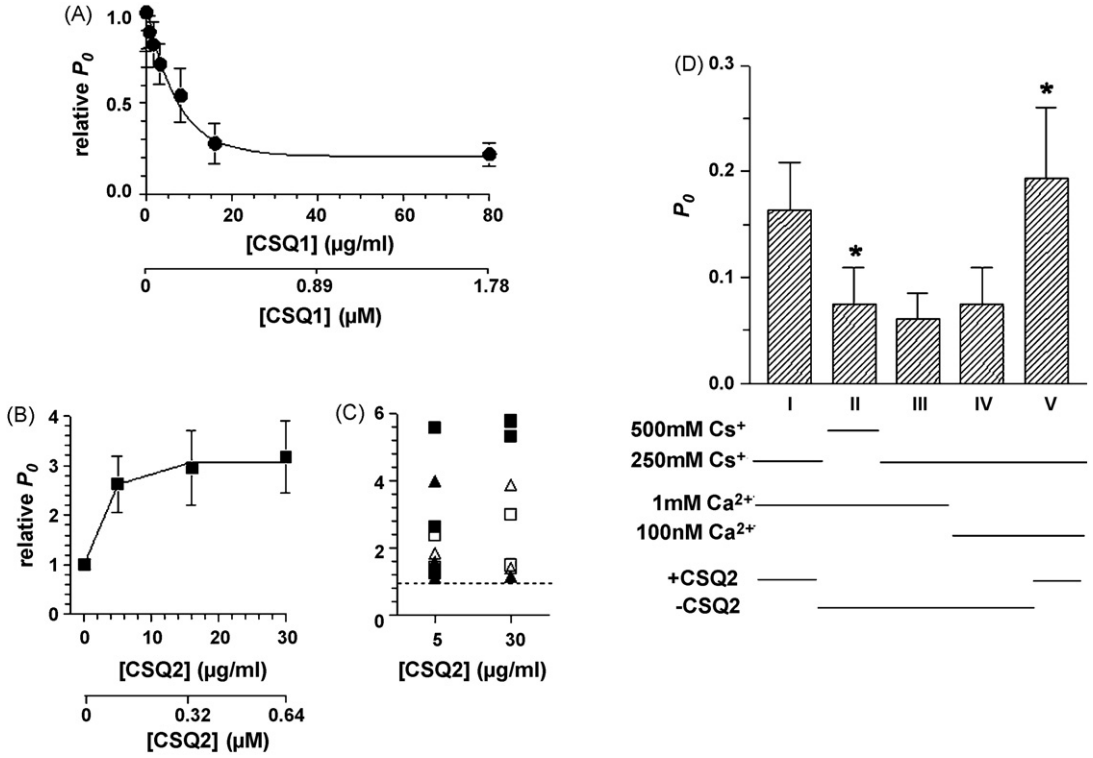

Fig. 6. Concentration-dependence of CSQ effects on RyR channels and the action of CSQ2 on RyR2 in the presence of low luminal Ca^{2+} (100 nM). (A and B) The average relative open probability (relative P_o) of RyR1 as a function of luminal CSQ1 concentration (A, $n = 3$–10) or of RyR2 as a function of CSQ2 concentration (B). Data with 16 μg/ml CSQ2 were obtained only with CSQ2 isolated from sheep heart after endogenous CSQ2 dissociation with high ionic strength ($n = 9$). The averages shown for 5 μg/ml and 30 μg/ml CSQ2 were obtained combined from experiments with sheep heart CSQ2 following high ionic strength treatment ($n = 4$) or recombinant human CSQ2 following endogenous CSQ2 dissociation by exposure to 5 mM luminal Ca^{2+} [13] ($n = 2$). The mM concentration of CSQ2 was calculated using scansite.miy.edu/calc-MW_pi.html. Sheep CSQ2 has not been sequenced, therefore the molecular weight (MW) is given as the average of MW dog, rabbit, rat and human CSQ2 (47360), with a range of 46300–48100). (C) Individual data points for sheep heart CSQ2 after high ionic strength treatment (filled symbols) or recombinant human CSQ2 after 5 mM Ca^{2+} treatment (open symbols) at +40 mV (squares) or −40 mV (triangles). (D) Average RyR2 P_o ($n = 9$) at +40 mV and −40 mV at the start of the experiment (I), after 5 min exposure to 500 mM *trans* (II), after return to 250 mM *trans* Cs^+ (III), after lowering the *trans* [Ca^{2+}] to 100 nM (IV) and finally after adding exogenous CSQ2 to the *trans* chamber (V). Asterisks (*) indicate significant differences from the value in the preceding condition.

CSQ2 were obtained in two ways. Either endogenous CSQ2 was dissociated with high ionic strength and the exogenously added CSQ2 isolated from sheep heart (filled symbols, Fig. 6C), or endogenous CSQ2 dissociated by exposure to 5 mM Ca^{2+} and the exogenous CSQ2 added back was recombinant human CSQ2 (open circles, Fig. 6C) which was purified using a very different procedure (see Section 2). The overlapping points suggest that the activation was not dependent on the dissociation procedure and was not altered by the CSQ2 purification procedure or the species from which CSQ2 was obtained.

As discussed above, one possibility is that a fraction of CSQ2 binds reversibly to RyR2, while we have evidence only for irreversible binding of CSQ1 polymers to RyR1 when the luminal [Ca^{2+}] is 1 mM. Thus the binding of CSQ1 to RyR1 is likely to be more correctly a time-dependent function of CSQ1 concentration. We know that at 16 μg/ml CSQ1 takes 3–5 min to inhibit RyR1, and the effects of lower concentrations were measured within a similar time frame because of the finite life time of the bilayer. The time- and concentration-dependence of CSQ1 and CSQ2 effects on RyR1 and RyR2 will need to be investigated in greater detail.

3.6. Regulation of RyR2 by CSQ2 with low luminal Ca^{2+}

In contrast to the increase in RyR2 activity that we see with addition of CSQ2 to RyR2 in the presence of 1 mM luminal Ca^{2+}, it has been suggested that CSQ2 inhibits RyR2 when added at a luminal Ca^{2+} concentration of 20 μM [2]. Therefore we examined the effect of CSQ2 on RyR2 under our conditions with low luminal Ca^{2+}. This was examined in RyR2 channels after treatment with 500 mM Cs^+ to dissociate endogenous CSQ2 (Fig. 6D). There was a delayed 2-fold decrease in channel activity after increasing Cs^+ to 500 mM which was likely due to CSQ2 dissociation from RyR2. Restoration of 250 mM Cs^+ did not reverse this decrease in activity indicating that it was not simply due to the change in ionic strength. In addition, there was no significant change in activity when *trans* [Ca^{2+}] was lowered to 100 nM. Finally, addition of 16 μg/ml CSQ2 resulted in a significant >2-fold activation of RyR2. Thus, in our hands, CSQ2 activates native RyR2 channels with both 1 mM and 100 nM luminal Ca^{2+}. Since CSQ2 is mostly a monomer in the presence of both 1 mM Ca^{2+} and lower Ca^{2+} concentrations, it was not surprising that its action on RyR2 would be the same at 1 mM and lower Ca^{2+} concentrations. This result is different from that obtained with purified RyR2/triadin complex [2] and may be due to our use of native RyR2 channels with the full compliment of associated proteins.

We have recently shown that CSQ1 monomers activate RyR1 at low luminal Ca^{2+} (100 nM), as opposed to the inhibition of RyR1 by CSQ polymers at 1 mM Ca^{2+} [14], indicating that the CSQ1/RyR1 interaction is strongly Ca^{2+}-dependent. In stark contrast, we show here that the excitatory action of CSQ2 on RyR2 is not influenced by luminal Ca^{2+}.

3.7. Isoform specificity of CSQ interactions with RyR channels

We next determined whether RyR1 inhibition by CSQ1, or RyR2 activation by CSQ2 (in the presence of 1 mM Ca^{2+}), was specific for the RyR isoform. The effect of CSQ2 on RyR1 was examined after the

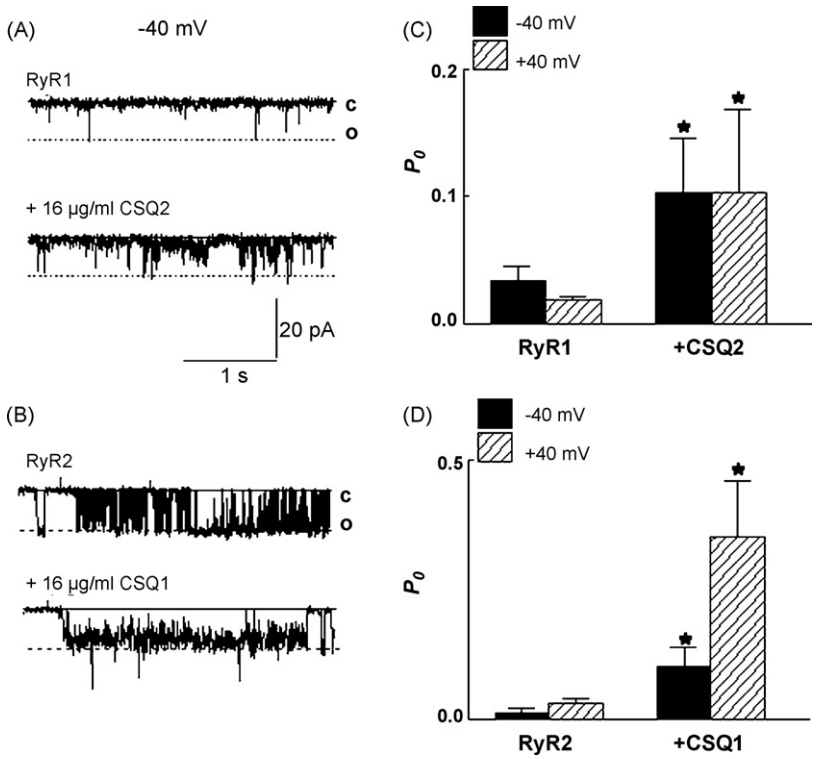

Fig. 7. Isoform specificity in CSQ regulation of RyRs. (A and B) Records of 3 s of channel activity at −40 mV. Single channel opening is downward from zero current (c) to maximum open conductance (o) at −40 mV with 500 nM *cis* Ca^{2+}. Upper control records were obtained with 1 mM *trans* Ca^{2+} following exposure of RyR1 (A) or RyR2 (B) to 100 nM *trans* Ca^{2+}. The lower records were obtained after addition of 16 μg/ml CSQ2 (A) or CSQ1 (B). (C) Average P_o recorded before (RyR1) and after (+CSQ2) CSQ2 addition ($n = 8$). (D) Average P_o recorded before (RyR2) and after (+CSQ1) CSQ2 addition ($n = 5$). Asterisks (*) show significant differences from values before CSQ addition.

RyR had been treated with a low Ca^{2+} solution (as above) to depolymerize endogenous CSQ1. The Ca^{2+} concentration in the *trans* chamber was restored to 1 mM and CSQ2 was added and found to activate RyR1 (Fig. 7A and B), showing that CSQ2 is an activator of both RyR1 and RyR2. The effect of CSQ1 on cardiac RyR2 was examined in the same way and found to activate RyR2 (Fig. 7C–D), in contrast to the inhibition that CSQ1 imposed on RyR1. These data show that the skeletal RyR1 is sensitive to the CSQ isoform, being inhibited by CSQ1 or activated by CSQ2. In contrast, the cardiac isoform of the RyR does not discriminate between the CSQ isoforms, with both CSQ1 and CSQ2 increasing P_o. Interestingly, slow-twitch skeletal muscle expresses RyR1 and both CSQ1 and CSQ2 [9,10]. In this situation the overall regulation of RyR1 by CSQ would depend on the relative amounts of CSQ1 and CSQ2 expressed in a particular fiber.

3.8. General conclusions

There are several points that we have kept in mind in interpreting the data and developing a model for protein/protein interactions between CSQ and RyR channels (e.g. Fig. 5C). The first is that CSQ monomers bind strongly to triadin/junctin at low luminal Ca^{2+} (100 nM and 100 μM) in heart and skeletal preparations [20,37] and CSQ2 monomers appear to be the key RyR2 regulating entity as suggested in [4]. This led to the assumption that exposure to low luminal Ca^{2+} leaves a CSQ monomer attached to the RyR complex. Secondly when luminal [Ca^{2+}] reaches 3–10 mM (exceeding the physiological 1 mM) CSQ becomes super compacted and no longer binds to triadin/junctin [20,37], so that the effect of exposure to luminal Ca^{2+} >3 mM on RyR activity cannot be interpreted in terms of CSQ regulation unless the duration of exposure is carefully considered. Finally, CSQ can activate purified RyRs by binding directly to the protein [13,39]. The following model takes into account the data presented here and published by us and others.

1. In the presence of [Ca^{2+}] between 100 nM and 100 μM, CSQ1 and CSQ2 are monomeric.
2. When the luminal [Ca^{2+}] is 100nM–100 μM, exogenous CSQ1 or CSQ2 added to the luminal solution in lipid bilayer studies activate both RyR1 and RyR2. This may be due to CSQ binding directly and reversibly to the RyR.
3. There are substantial differences between cardiac and skeletal CSQ when exposed to the resting physiological luminal [Ca^{2+}] of 1 mM. CSQ1 is mostly polymerized, while CSQ2 is mostly a monomer.
4. There are very different effects of cardiac and skeletal CSQ on RyR1 and RyR2 channel activity when exogenous CSQ is added to the luminal side of RyRs with 1 mM luminal Ca^{2+}. RyR1 is inhibited by the CSQ1 polymer. On the other hand, RyR1 and RyR2 are activated by the CSQ2 monomer. Once again the activation of RyR2 may be through CSQ2 directly binding to RyR2.
5. Together these data suggest that under *in vivo* conditions (1 mM luminal Ca^{2+}) CSQ2 increases the open probability of RyR2 and RyR1, while CSQ1 reduces the activity of RyR1.

3.9. Physiological significance

Our results suggest several important differences between the roles of the two CSQ isoforms in modifying RyR activity *in vivo*. CSQ2 would help maintain maximal efflux of Ca^{2+} from the SR in the heart during systole by increasing the gain of RyR2. The heart is geared for the SR to lose much of its Ca^{2+} (24–63% fall in free Ca^{2+} [31,32]) and refill during each contraction cycle. Activation of RyR2 by the CSQ2 monomer is not Ca^{2+} dependent and is retained through the large fluctuations in luminal Ca^{2+} which occur with every contraction cycle. On the other hand in skeletal muscle a much smaller fraction of Ca^{2+} is released with each action potential and twitch (~1% fall in free Ca^{2+} [38]) and release is maintained over a range of action

potential frequencies and stimulation durations. CSQ1 facilitates this function by acting as a break on Ca^{2+} release. RyR1 regulation by CSQ1 is maintained during the rapid fluctuations in luminal Ca^{2+} following a single action potential or a short train of action potentials, because luminal Ca^{2+} mostly does not fall sufficiently to initiate depolymerization and the polymer is slow to dissociate [3]. However prolonged high frequency stimulation or exposure to RyR agonists can severely deplete Ca^{2+} in skeletal SR [38] and the CSQ1 polymer may dissociate and relieve the inhibitory regulation of RyR1.

In addition to these direct effects on the RyR activity, CSQ confers a specific luminal Ca^{2+}-dependence on the RyR1 and RyR2. When the CSQ1 polymer is associated with RyR1, channel activity does not change or decreases slightly when luminal Ca^{2+} is lowered from 1 mM to 100 μM and activity increases when luminal Ca^{2+} is raised from 1 mM to 5 mM [3,19]. This is in contrast to a rise in RyR1 activity in the absence of CSQ1 [3]. RyR2 activity is similarly reduced when $[Ca^{2+}]$ is lowered from 5 mM to 20 μM in the presence of CSQ2 [2], although the degree of CSQ2 association with RyR in the presence of 5 mM Ca^{2+} is unclear. When examined in finer detail, the effect Ca^{2+} on RyR2 in the presence of CSQ2 appears biphasic, with low channel activity between 1 mM and 10 mM (although CSQ2 may be associated), higher activity between 100 μM and 1 mM and decreasing again with <100 μM Ca^{2+} [4,40]. In the absence of CSQ2, RyR2 activity appears not to change with luminal Ca^{2+} [2,4]. The precise luminal Ca^{2+}-dependence varies between laboratories and experimental conditions. Our data in Fig. 4 show no change in activity when Ca^{2+} is lowered from 1 mM to 10 μM, which may have been due to these Ca^{2+} concentrations bracketing the activating concentrations. Although the precise responses of RyR1 and RyR2 to changes in luminal Ca^{2+} concentration vary, CSQ in both skeletal and cardiac muscle appears to facilitates Ca^{2+} release when the store is full but not when luminal Ca^{2+} falls.

3.10. Disruption to Ca^{2+} signaling caused by CSQ2 mutations or knockout

The role of CSQ in regulation of RyR activity in skeletal and cardiac muscle *in vivo* is incompletely understood. Single CSQ2 mutations in mice or lack of CSQ2 expression lead to a catecholaminergic polymorphic ventricular tachycardia (CPVT) phenotype or an altered capacity for luminal Ca^{2+} to regulate RyR2 [4,6,31,41,42]. The knockout and mutation studies show that CSQ is essential for the normal structure and function of muscle fibers. However the changes in Ca^{2+} regulation are not easily reconciled with results obtained with the isolated proteins, most likely because Ca^{2+} regulation is also affected by changes in expression of other proteins, changes in Ca^{2+} stored in the SR and changes in the structure of the SR. Contraction is preserved in the absence of CSQ1 and CSQ2 through major adaptive changes in structure and protein composition [6,43]). CSQ2 knockout [6] and the CPVT mutations D307H [42] and R33Q [31] result in an increases diastolic Ca^{2+} leak, spontaneous Ca^{2+} release and Ca^{2+} oscillations under basal conditions and with catecholamine stimulation, which presumably underlies CPVT. At the same time, there is a reduction in the amplitude of caffeine-induced or I_{Ca}-induced Ca^{2+} transients with the D307H mutation [42,44] and contraction with CSQ2 knockout (see records in Fig. 4 of [6]). These changes could be caused by any, or a combination, of changes in several factors including CSQ2, SR volume [6], expression of RyR2 and calreticulin (which increase in CSQ2 knockout and the D307H mutation [45]), triadin and/or junctin expression (which is reduced with CSQ2 knockout and the R33Q mutation [6,46]) or binding of mutant CSQ2 to triadin and junctin (which is reduced with the D307H or truncation mutations [31,41]). Finally Ca^{2+} binding capacity of CSQ2 and its ability to dimerize or polymerize with low ionic strength or high Ca^{2+} is disrupted to a greater or lesser extent in the D307H, R33Q and L167H mutations [5,41,47]. Since Ca^{2+} regulation is a complex function of the amount of Ca^{2+} in the SR, the open probability of RyR2 channels, the amounts of SR and number of RyR channels, these whole cell studies do not reveal the molecular nature of interactions between CSQ2 and RyR2.

Several of the results obtained with the knock out and CPVT mutations in CSQ2 are consistent with the activation of RyR2 channels by CSQ2 (this manuscript and [4]). These include the depression of I_{Ca} and caffeine -activated Ca^{2+} release and contraction [6,41,42], although this depression could also be explained by reduced amounts of Ca^{2+} in the SR [31]. Also consistent with the concept that wild-type CSQ2 activates RyR2, is the increased amplitude of spontaneous Ca^{2+} release events that occurs with CSQ2 over expression [31,44] and the decreased amplitude with the deletion mutant and D307H that prevents CSQ2 binding to triadin and regulating RyR2 [31,44].

Conflict of interest

None of the authors have any conflict of interest that might bias the work.

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